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ANALYSIS

VALIDATION OF A HIGH–PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR PHARMACOKINETIC EVALUATION OF PENTOXIFYLLINE AND LISOFYLLINE IN RAT SERUM AND TISSUES

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Abstract: The aim of this paper was to validate an analytical method for the simultaneous determination of PTX and its active metabolite (-)-(R)-M1 in rat serum and some tissues using a high-performance liquid chromatography method with ultraviolet detection (HPLC-UV). The specificity, linearity, precision, accuracy, recovery, lower limit of detection, lower limit of quantification and stability study were successively conducted according to GLP procedures. HPLC separation of all compounds was carried out on a normal-phase ChiralPak AD column (250 mm × 4.6 mm i.d., 5 mm), using, as a mobile phase, a mixture of hexane and 2-propanol (84:16, v/v) containing 0.01% of diethylamine with a flow rate of 1.5 mL × min^{-1}. The calibration curves from all studied matrices were linear across the concentration range from 0.01 to 100 mg × mL^{-1} with a lower limit of quantification of 0.01 μg × mL^{-1} for all analytes. The application of the assay to a pilot pharmacokinetic study and tissue distribution of the compounds in rats after intraperitoneal dosing of 50 mg × kg^{-1} of PTX was described. Significant ($p < 0.05$) differences between serum and tissue levels of PTX, (-)-(R)-M1 and (+)-(S)-M1 were observed.

Keywords: lisofylline, pentoxifylline, HPLC, method validation, tissues, enantioselectivity

Pentoxifylline (PTX) is one of several methylxanthine compounds used as a hemorheologic agent for the treatment of peripheral vascular disease and intermittent claudication. In addition to its ability to improve microcirculation, PTX also proves an anti-tumor necrosis factor-alpha (TNFα) action and immunomodulatory properties (1). *In vivo*, PTX is reduced by carbonyl reductase to a pharmacologically active stereoisomeric hydroxyl metabolite M1 (Figure 1). This biotransformation process is rapidly reversible and takes place both in erythrocytes (2) and liver microsomes and cytosol (3) and probably also in some other tissues, such as lung and kidney.

The biotransformation of PTX to M1 is highly stereoselective in favor of the (+)-(S)-M1 enantiomer formation, in both the *in vitro* and *in vivo* study (4). The less favored and biologically active (-)-(R)-M1 enantiomer (lisofylline) accounts only for 5-10% of the total metabolite M1 in blood (3). Previously published analytical data indicate an increase of the formation of (-)-(R)-M1 from PTX in, for example, the presence of ciprofloxacin (5). In human liver microsomes, enantiomer (+)-(S)-M1 is exclusively converted to PTX, whereas approximately 45% of (-)-(R)-M1 oxidation is accounted for by the formation of PTX and the balance of aliphatic diols (6). CYP1A2 is the highest affinity enzyme catalyzing the biotransformation of (-)-(R)-M1 to PTX (7), whereas CYP3A4 and CYP2A6 isozymes catalyze the metabolism of (-)-(R)-M1 to a diol (6).

Lisofylline, originally developed as a novel anti-inflammatory compound which reduces inflammatory cytokine production and its activity, improves beta-cell mitochondrial metabolism, regulates immune activities and reduces cellular damage due to ischemic reperfusion, hypoxia or autoimmune disease (8). It has been proven that the (-)-(R)-M1 enantiomer is several hundred times more effective than its parent compound, PTX, at inhibiting the activity of inflammatory cytokines (9). Lisofylline modulates stress associated changes in lipid metabolism and has been tested to modify the toxicity for patients undergoing intensive dose cytotoxic therapy and to prevent multiorgan failure in acute respi-

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ratory distress syndrome (10). It has been reported that (-)-(R)-M1 can decrease the dysfunction of rat pancreatic islets caused by interleukin-1 beta (IL-1β) (11).

Several analytical methods using gas chromatography (12, 13) and reversed-phase HPLC with ultra-violet detection (14, 15) have been developed for the determination of PTX and its major metabolites in plasma and also for the separation of the M1 enantiomers after chiral derivatization (4). Recently, a liquid chromatography-tandem mass spectrometry method has been described to determine PTX and (-)-(R)-M1 in human and rabbit plasma (16). The latter method has provided good results, but it was achiral and involved expensive equipment not easily available.

The purpose of this study was to develop and validate according to validation procedure parameters and acceptance criteria based on USP XXIII guidelines and FDA guidance (17), a direct, enantioselective high-performance liquid chromatography method with ultra-violet detection for the simultaneous quantification of PTX and (-)-(R)-M1 enantiomer in rat serum and some tissues.

EXPERIMENTAL

Materials and reagents

Pentoxifylline, 3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydropurine-2,6-dione, was obtained from Sigma (St. Louis, MO, USA), and metabolite M1, 3,7-dimethyl-1-(5-hydroxyhexyl)-3,7-dihydropurine-2,6-dione, lisofylline, (-)-(R)-3,7-dimethyl-1-(5-hydroxyhexyl)-3,7-dihydropurine-2,6-dione and the internal standard, 3,7-dihydro-1,3-dimethyl-7-(2-chloroethyl)-xanthine, were supplied by the Department of Chemical Technology and Biotechnology of Drugs (Faculty of Pharmacy, Jagiellonian University, Medical College, Kraków).

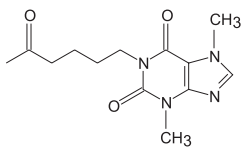
HPLC grade hexane, 2-propanol, diethylamine, methanol, acetonitrile, hydrochloric acid, dichloromethane, chloroform, dihydropotassium phosphate, orthophosphoric acid and natrium chloride were purchased from Merck (Darmstadt, Germany).

Pooled control serum and tissue samples were obtained from adult male Wistar rats Krf:(WI)WV (Charles River Laboratory, Germany), weighing 200 to 220 g. The study was approved by the Institutional Animal Care and Ethics Committee. Rats were injected intraperitoneally with thiopental (60 mg × kg^{-1}), and blood samples were collected from the left jugular vein. The serum was separated by centrifugation (1500 g, 10 min) and stored at – 30°C until used. The tissue samples (liver, kidney, lung, heart and spleen) were stored at – 80°C until assayed.

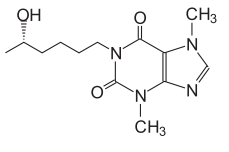
Instrumentation and chromatographic conditions

The method validation was performed on a ThermoSeparation Products HPLC system (San Jose, CA, USA) with a variable wavelength UV-visible spectrophotometric detector (Spectra 100). The elution profiles in the HPLC separations were monitored by absorbance set at 275 nm at 0.02 AUFS. Injections were done using a Rheodyne 7125 manual injector (Alltech Associates, Deerfield, IL, USA) fitted with a 50 mL sample loop (Supelpro, Supelco, Germany). Chromatograms were recorded and integrated with a model SP 4400 ChromJet integrator (San Jose, CA, USA).

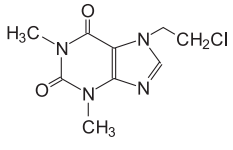
Analyses were performed on an amylose ChiralPak AD (250 mm × 4.6 mm i.d., 5 mm) analytical column (Daicel Chemical Industry, Tokyo, Japan) protected by a Supelcosil LC-Si guard column (Supelco, Germany) and operated at room temperature. Optimal separation of the compounds was achieved using a normal mobile phase containing *n*-hexane and 2-propanol (84:16, v/v) with 0.01% diethylamine and pumped through the system at a flow rate of 1.5 mL × min^{-1}.



PTX - 3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydropurine-2,6-dione



Lisofylline -(-)-(R)-1-(5-hydroxyhexyl)-3,7-dimethylxanthine



Internal standard -3,7-dihydro -1,3-dimethyl-7-(2-chloroethyl)-xanthine

Figure 1. Structures of pentoxifylline, lisofylline and internal standard.

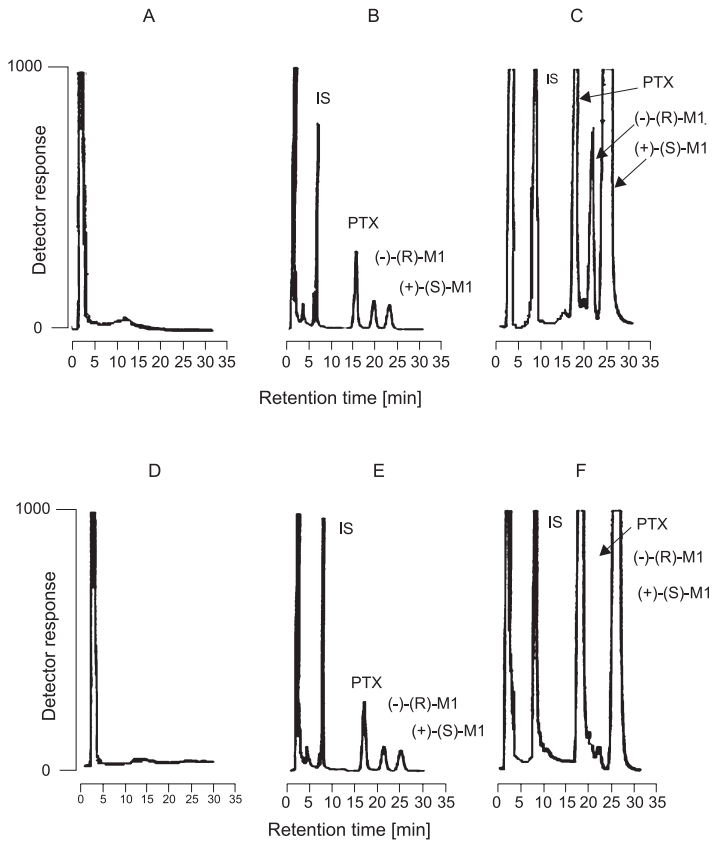


Figure 2. Representative chromatograms: drug-free rat serum sample (A), rat serum sample containing IS, PTX and M1 at a concentration of 1 mg × mL^{-1} (B), serum sample from a rat receiving PTX at intraperitoneal dose of 50 mg × kg^{-1} (C), drug-free rat kidney sample (D), rat kidney sample containing IS, PTX and M1 at a concentration of 1 mg × mL^{-1} (E), kidney sample from a rat receiving PTX at intraperitoneal dose of 50 mg × kg^{-1} (F).

Preparation of standard solutions, calibration curves and quality control samples

Stock solutions of PTX and (-)-(R)-M1 at a concentration of 1000 mg × mL^{-1} and its working solutions at concentrations of 0.1, 1, 2.5, 10, 25, 50, 500 and 1000 μg × mL^{-1} were prepared in methanol. Similarly, a stock solution (1000 mg × mL^{-1}) and a working solution (50 mg × mL^{-1}) of internal standard was also prepared in methanol. Stock solutions and all working standards were stored at 4°C.

Calibration curves were constructed by supplementing 50 mL of the appropriate working solution to 0.45 mL of drug-free serum samples or to 0.45 mL of drug-free homogenate tissue samples. Concentrations of the analytes were 0.01, 0.1, 0.25, 1, 2.5, 5, 50 and 100 mg × mL^{-1}. To all samples 50 mL of the internal standard working solution was added. For serum and tissue samples, before sample pretreatment, the mixture was vortexed briefly and then incubated at 4°C for 15 min.

Quality control (QC) samples containing the analytes were prepared at four different concentrations along the calibration range (low at 0.015 mg × mL^{-1}, medium at 0.5 mg × mL^{-1} and 4 mg × mL^{-1} and high at 80 mg × mL^{-1}) by spiking drug-free serum and tissue samples with the analytes, as described above, and kept frozen at – 30 and – 80°C, respectively.

Sample preparation procedure in the animal's treatment group

Both serum and tissue sample extraction procedure involved a liquid-liquid extraction. Before use, tissue samples were thawed, an aliquot of 200 mg was weighed and placed in a glass mortar and pestle tissue grinder. The tissues were homogenized with 0.5 mL of phosphate buffer (pH 7.4), and 0.5 mL of homogenates were transferred to new glass centrifuge tubes and spiked with 50 μL of the internal standard working solution (50 mg × mL^{-1}). Similarly,

Table 1. Chromatographic separation of the compounds in rat serum (n = 3)

Compound			
Parameters ± SD	IS	PTX	(-)-(R)-M1
k'	1.93 ± 0.016 R.S.D. = 0.83%	5.18 ± 0.012 R.S.D. = 0.22%	6.9 ± 0.04 R.S.D. = 0.59%
α_1 α_2	2.67 ± 0.025 1.33 ± 0.0057	R.S.D. = 0.94% R.S.D. = 0.58%	
R_s	1.94 ± 0.06	R.S.D. = 2.93%	

0.5 mL of rat serum was also spiked with 50 µL of the internal standard working solution. Serum and tissue samples were acidified with 0.1 mL of hydrochloric acid (0.1 M), shaken with 3 mL of a mixture of dichlorometane – chloroform (50:50, v/v) for 20 min and then centrifuged (2000 g, 15 min). Two mL of the organic phase was evaporated to dryness under a stream of nitrogen. The dried residue was reconstituted in 100 µL of the mobile phase and a 50 µL volume was injected into the HPLC column.

Stability study

Stability of the analytes was determined periodically by injecting replicate preparations of processed samples. The peak areas of the analyte and IS obtained in the initial cycle were used as the reference to determine the stability at subsequent points. The stability of each compound in the matrix over 6 h (bench-top) was determined at an ambient temperature (25 ± 3°C) at concentrations of three QC samples (0.015, 0.5 and 4 µg × mL^{-1}). Freezer stability of the compounds in rat serum and tissues was assessed by analyzing the QC samples stored at – 30 and – 80°C, respectively, for four weeks. The stability of each compound was also assessed after three freeze/thaw cycles using three levels of QC samples. Serum and tissue samples were stored at – 30 and – 80°C, respectively. Between freeze/thaw cycles, samples were thawed by allowing them to stand at room temperature for approximately 1 h. The samples were then returned to the freezer.

Samples were considered to be stable if assay values were within the acceptable limits of accuracy and precision.

Assay validation

Specificity of the method was investigated by analysis of six different batches of pooled blank rat serum and tissue homogenate samples, to determine whether endogenous constituents coeluted with the different analytes. The retention times of endogenous compounds in the matrix were compared with that of the compounds of interest.

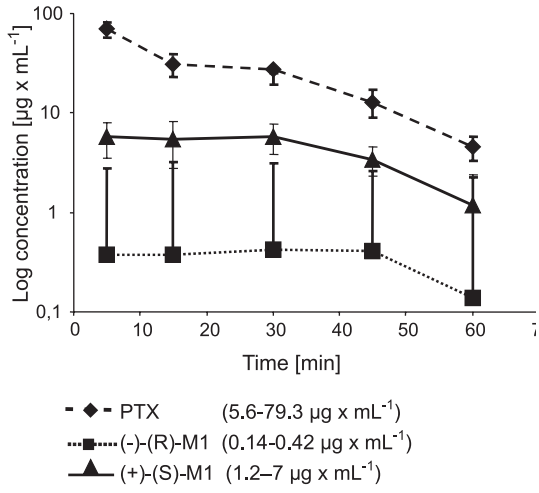


Figure 3. Serum concentration of PTX, (-)-(R)-M1 and (+)-(S)-M1 after intraperitoneal administration of PTX at a dose of 50 mg × kg^{-1} in rat (semilogarithmic plot).

Replicate injections (n = 6) of a solution containing the racemic mixture of M1 at a concentration of 1 mg × mL^{-1} have shown two different peaks, each of them corresponding to an enantiomer, with an area ratio of 0.998 ± 0.08.

Standard calibration curves were obtained from an unweighted, least-squares linear regression analysis of the data. The slope and intercept of the calibration graphs were determined through linear regression of the drug to an internal standard peak area ratio versus drug concentration plot (formula: y = ax + b; where x = concentration and y = peak area ratio). Individual peak area ratios were then interpolated on the calibration graphs to determine the values of the concentration found (back-calculated concentration) as compared to the concentration added.

Quality of fit was evaluated by comparing back-calculated concentrations to the nominal ones. The "lack of fit" test was used to confirm the linear-

Table 2. Assessment of intra- and inter-day precision, accuracy and recovery of the method for PTX, (n = 5 to 6)

Nominal concentration [μg × mL^{-1}]* [μg × g^{-1}]**	Mean Equation of regression	Intraday reproducibility			Interday reproducibility			Recovery [%]
		back-calculated concentration [μg × mL^{-1}]* [μg × g^{-1}]**	Precision [%]	Accuracy [%]	Mean back-calculated concentration [μg × mL^{-1}]* [μg × g^{-1}]**	Precision [%]	Accuracy [%]	
Serum	y = 0.002x -							
0.015	0.008	0.0135 ± 0.002	14.8	90	0.0138 ± 0.002	14.5	92	96.3 ± 4.3
4		4.12 ± 0.12	2.9	103	4.21 ± 0.18	4.3	105	91.7 ± 12.5
80	R^2 = 0.977	84.7 ± 2.6	3.1	106	82.8 ± 6.8	8.2	103.5	112.3 ± 5.6
Liver	y = 0.002x -							
0.015	0.014	0.0138 ± 0.002	14.5	92	0.0145 ± 0.001	6.9	96.7	91.2 ± 3.6
4		3.93 ± 0.14	3.6	98.3	4.17 ± 0.12	2.9	104.3	96.3 ± 11.2
80	R^2 = 0.986	87.5 ± 3.5	4	109.4	90.4 ± 4.1	4.5	113	89.5 ± 7.8
Lung	y = 0.002x -							
0.015	0.005	0.0144 ± 0.002	13.9	96	0.0137 ± 0.002	14.6	91.3	89.7 ± 3.3
4		4.22 ± 0.17	4	105.5	3.88 ± 0.15	3.9	97	95.2 ± 11.6
80	R^2 = 0.981	91.4 ± 4.6	5	114.3	86.7 ± 7.8	9	108.4	97.5 ± 8.5
Kidney	y = 0.002x -							
0.015	0.0017	0.0158 ± 0.002	12.7	105.3	0.0142 ± 0.002	14.1	94.7	85.4 ± 4.7
4		4.17 ± 0.21	5	104.3	3.57 ± 0.19	5.3	89.3	111.7 ± 12.5
80	R^2 = 0.982	88.3 ± 2.8	3.2	110.4	89.2 ± 8.5	9.5	111.5	110.4 ± 7.8
Heart	y = 0.002x -							
0.015	0.021	0.0172 ± 0.002	11.6	114.7	0.0139 ± 0.002	14.4	92.7	86.7 ± 4.1
4		3.81 ± 0.12	3.2	95.3	4.23 ± 0.18	4.3	105.8	90.8 ± 15.8
80	R^2 = 0.982	89.1 ± 7.5	8.4	111.4	91.5 ± 6.4	7	114.4	92.3 ± 8.3
Spleen	y = 0.002x -							
0.015	0.0024	0.0165 ± 0.002	12.1	110	0.0138 ± 0.002	14.5	92	87.8 ± 3.1
4		3.86 ± 0.13	3.4	96.5	4.27 ± 0.21	4.9	106.8	88.4 ± 16.6
80	R^2 = 0.988	91.4 ± 4.5	4.9	114.3	90.4 ± 5.3	5.9	113	106.5 ± 9.4

* serum; * * tissue

Table 3. Assessment of intra- and interday precision, accuracy and recovery of the method for (-)-(R)-M1, (n = 5 to 6)

Nominal concentration [μg × mL^{-1}]* [μg × g^{-1}]**	Mean Equation of regression	Intraday reproducibility			Interday reproducibility			Recovery [%]
		back-calculated concentration [μg × mL^{-1}]*	Precision [%]	Accuracy [%]	Mean back-calculated concentration [μg × mL^{-1}]*	Precision [%]	Accuracy [%]	
Serum	y = 0.003x -							
0.015	0.004	0.0138 ± 0.002	14.5	92	0.016 ± 0.002	12.5	107	92.2 ± 3.3
4		3.82 ± 0.18	4.7	95.5	4.23 ± 0.16	3.8	106	109.7 ± 15.5
80	R^2 = 0.982	87.4 ± 9.3	10.6	109	89.2 ± 6.6	7.4	112	88.5 ± 14.2
Liver	y = 0.003x -							
0.015	0.008	0.0136 ± 0.002	14.7	90.7	0.0144 ± 0.002	13.8	96	90.2 ± 4.6
4		4.44 ± 0.23	5.2	111	3.87 ± 0.12	3.1	96.8	109.3 ± 14.2
80	R^2 = 0.974	91.2 ± 7.5	8.2	114	85.7 ± 7.3	8.5	107	91.4 ± 12.7
Lung	y = 0.003x -							
0.015	0.005	0.014 ± 0.002	14.3	93.3	0.017 ± 0.002	11.8	113	87.5 ± 4.3
4		4.52 ± 0.19	4.2	113	3.88 ± 0.17	4.4	97	107.2 ± 11.6
80	R^2 = 0.988	89.5 ± 7.9	8.8	112	90.2 ± 7.9	8.8	113	90.7 ± 10.5
Kidney	y = 0.003x -							
0.015	0.002	0.017 ± 0.002	11.8	113	0.0132 ± 0.002	15.1	88	83.4 ± 3.7
4		3.88 ± 0.11	2.8	97	3.87 ± 0.18	4.7	96.8	111.3 ± 14.5
80	R^2 = 0.986	85.7 ± 6.8	7.9	107	89.2 ± 7.7	8.6	112	107.3 ± 12.4
Heart	y = 0.003x -							
0.015	0.004	0.0162 ± 0.002	12.3	108	0.0162 ± 0.003	12.3	108	88.6 ± 3.1
4		3.90 ± 0.11	2.8	97.5	4.26 ± 0.27	6.3	107	114.8 ± 17.8
80	R^2 = 0.978	90.3 ± 7.2	8	113	90.8 ± 9.1	10	114	89.2 ± 10.5
Spleen	y = 0.003x -							
0.015	0.006	0.0145 ± 0.002	13.8	93.3	0.0139 ± 0.002	14.4	92.7	92.1 ± 4.3
4		4.45 ± 0.13	2.9	111	4.28 ± 0.25	5.8	107	89.3 ± 18.6
80	R^2 = 0.983	82.8 ± 7.4	8.9	104	88.5 ± 3.6	4.1	111	107.5 ± 11.6

* serum; ** tissue

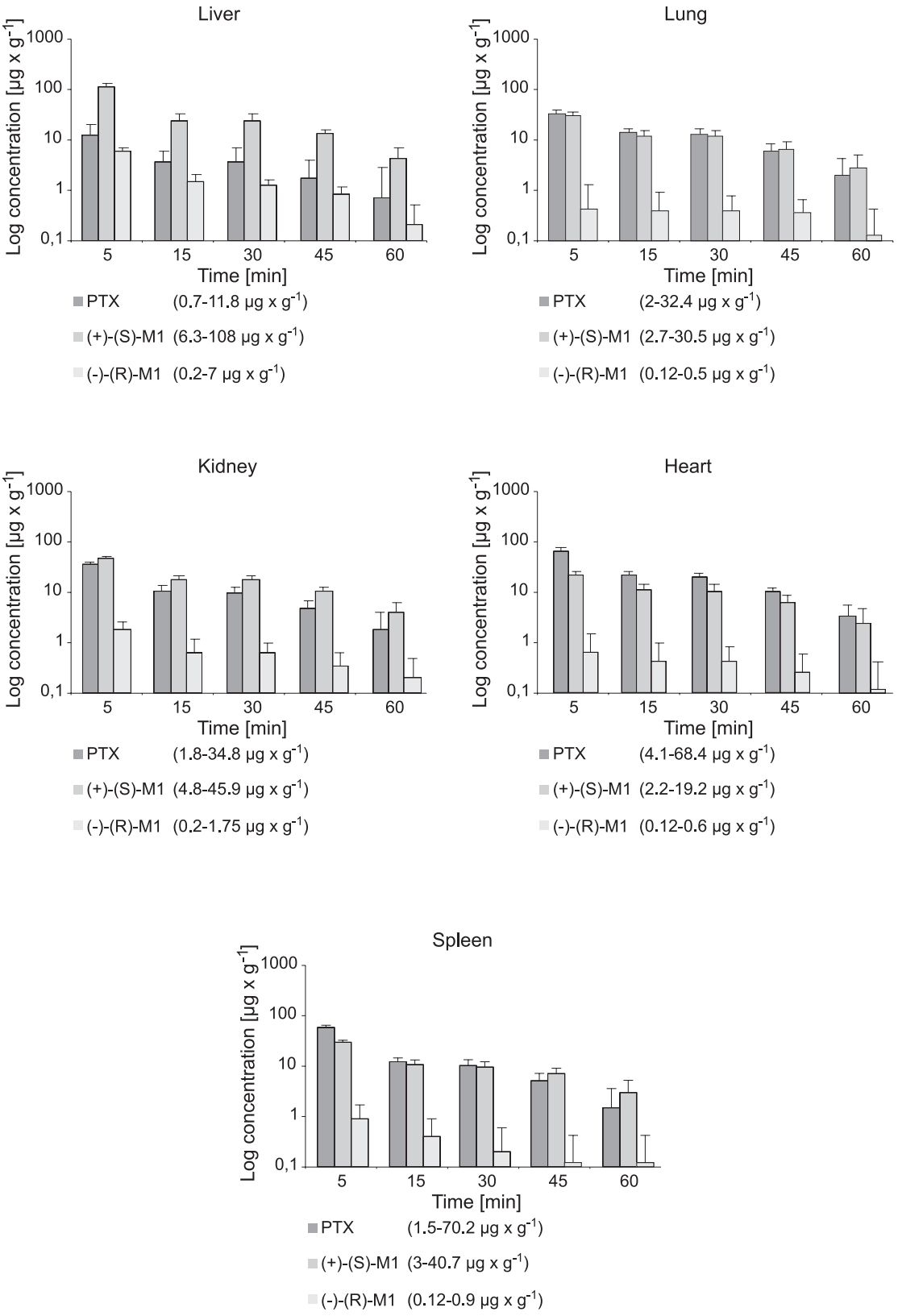


Figure 4. Tissue concentration of PTX, (+)-(S)-M1 and (-)-(R)-M1 after intraperitoneal administration of PTX at a dose of 50 mg × kg^{-1} in rat (semilogarithmic plot).

Table 4. Stability of PTX in rat serum, (n = 6)

Sample condition	Nominal concentration [μg × mL^{-1}]					
	0.015		0.5		4	
	Observed concentration [μg × mL^{-1}]	% Dev	Observed concentration [μg × mL^{-1}]	% Dev	Observed concentration [μg × mL^{-1}]	% Dev
Freshly prepared	0.0134	-10.7	0.542	8.4	4.27	6.8
6 h at room temperature	0.0142	-5.3	0.462	-7.6	3.86	-3.5
4 weeks at -30^{o}C	0.0164	9.3	0.544	8.8	4.13	3.3
Freeze/thaw cycle	0.0169	12.7	0.471	-5.8	3.71	-7.3

% Dev – percent error

Table 5. Stability of (-)-(R)-M1 in rat serum, (n = 5 to 6)

Sample condition	Nominal concentration [μg × mL^{-1}]					
	0.015		0.5		4	
	Observed concentration [μg × mL^{-1}]	% Dev	Observed concentration [μg × mL^{-1}]	% Dev	Observed concentration [μg × mL^{-1}]	% Dev
Freshly prepared	0.0139	-7.3	0.553	10.6	4.12	3
6 h at room temperature	0.0136	-9.3	0.461	-7.8	3.81	-4.8
4 weeks at -30^{o}C	0.0165	10	0.470	-6	4.109	2.7
Freeze/thaw cycle	0.0172	14.7	0.561	12.2	3.912	-2.2

% Dev – percent error

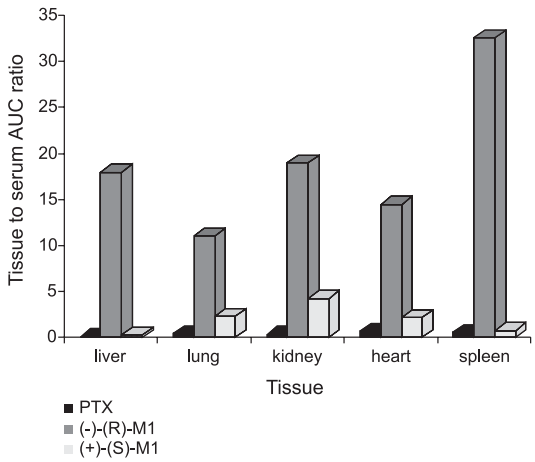


Figure 5. Tissue to serum AUC ratios for PTX, (-)-(R)-M1 and (+)-(S)-M1.

ity of the method.

Intra-day precision and accuracy of the assay were assessed by performing replicate (n = 5 to 6) analyses of QC samples in the serum and tissue against a calibration curve. The procedure was repeated on different days on the same spiked standards to determine inter-day repeatability.

Accuracy (percent of recovery) was evaluated as (mean found concentration/theoretical concentration) × 100. Precision was given by the percent relative standard deviation (R.S.D.).

Lower limit of detection (LLOD) was the lowest detected concentration of the analyte in a sample based on the signal to noise ratio 3:1. LLOD was determined by the analysis of a sample with known concentrations of the analyte and by establishing the minimum level at which the analyte can be detected.

Lower limit of quantification (LLOQ) was defined as the lowest drug concentration which can be quantitatively determined with a precision = 20%, and an accuracy of 100 ± 20%, and it was the lowest standard on the calibration curve.

Extraction recoveries of PTX and (-)-(R)-M1 in all studied matrices were evaluated at five to six replicates preceded by extraction procedure of spiking drug-free serum and drug-free tissue homogenate samples with low, medium and high QC levels of the analytes. The relative recovery was examined by comparing the peak areas of the extracted samples with equal amounts of solutions of the analytes injected directly into the HPLC column. Moreover, extraction recovery in the serum and tissues was calculated for the IS, at the concentration of 5 $\mu g \times mL^{-1}$.

A pilot pharmacokinetic study in rat

Using the chiral HPLC-UV method, a pilot pharmacokinetic profile and tissue distribution of PTX and enantiomers of its chiral metabolite M1 were studied, after intraperitoneal dosing of PTX in rats.

Male Wistar rats, 13-15 weeks of age and weighing between 200 and 220 g were used. They were kept under conditions of a constant temperature (21-25^{o}C) and a relative humidity of approximately 40-65% with a standard light/dark cycle. The animals were housed in stainless steel cages with suspended wire-mesh floors (maximum of 5 rats per cage). The animals were fasted overnight and then weighed. The rats had free access to water throughout the experimental period. PTX dissolved in 0.9% sterile isotonic saline at a dose of 50 $mg \times kg^{-1}$ was administered intraperitoneally. The rats were anaesthetized with thiopental at a dose of 60 $mg \times kg^{-1}$ and blood samples were collected from the left jugular vein to microfuge tubes at the following time intervals: 0 (predose), 5, 15, 30, 45, 60 and 120 min after dosing. Six rats at a time were sampled, and one sample per rat was collected. The serum and tissue samples were stored at – 30^{o}C and – 80^{o}C, respectively, until used.

RESULTS AND DISCUSSION

The previously published analytical methods allowed the measurement of the parent drug and its metabolites M1, M2, M4 and M5, as a racemic compounds in human (12), rabbit (18), mouse (19), dog (20) and horse (21) biological fluids, but determinations of these compounds, especially the enantiomers of metabolite M1 in rat biological matrices were rather underdone.

The present essay described a simple, reliable, sensitive and enantioselective method for direct analysis of PTX and (-)-(R)-M1 in rat serum and some tissues.

Representative chromatograms of serum and kidney tissue extracts are shown in Figure 2. On chromatograms from blank matrices, no endogenous interfering peaks were visible at the retention times of the analytes (Figure 2A and 2D). Under the chromatographic conditions described, the retention times from serum were 6.6 ± 0.1, 15.9 ± 0.3, 19.2 ± 0.2 and 25 ± 0.2 min for IS, PTX, (-)-(R)-M1 and (+)-(S)-M1, respectively. The retention times of the studied compounds from tissues were the same.

Good resolution of the analytes from endogenous compounds was obtained. The retention factors (k') were 1.93, 5.18 and 6.9 for IS, PTX and (-)-(R)-M1, respectively. The separation factors α_1 (PTX and IS separation) and α_2 [(-)-(R)-M1 and PTX separation] were 2.67 and 1.33, respectively. There was a clear resolution between the two enantiomers with the resolution factor (R_S) = 1.94 (Table 1).

Calibration curves for PTX and (-)-(R)-M1 in the serum and tissues were linear over of the studied concentration range (0.01 to 100 $mg \times mL^{-1}$). The equations of regression for PTX and for (-)-(R)-M1 in the serum and studied tissues are presented in Tables 2 and 3. The coefficients of correlation obtained using a linear regression analysis were higher than 0.974. No significant deviation from zero was found for the intercepts, and the "lack of fit" test showed no significant deviation from linearity. For each point of the calibration standards, the concentrations were back-calculated from the equation of the linear regression curves, and precision and accuracy values were calculated.

Precision data for intra- and inter-day PTX determination in all studied matrices were from 2.9 to 14.8% and from 2.9 to 14.6%, and for (-)-(R)-M1, they were from 2.8 to 14.7% and from 3.1 to 15.1%, respectively. Within-batch and between-batch accuracy values for PTX ranged from 90 to 114.7% and from 89.3 to 114.4%, and for (-)-(R)-M1, they were from 90.7 to 114% and from 88 to 114%, respectively (Tables 2 and 3).

Linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to zero (Student's *t*-test). The distribution of the residuals (difference between nominal and back-calculated concentrations) shows random variations, the number of positive and negative values being approximately equal. Moreover, they were normally distributed and centered around zero.

In all studied matrices, the lower limit of quantification of the method was found to be 0.01 mg × mL^{-1} for both PTX and (-)-(R)-M1 and was determined with high precision and accuracy, not exceeding 20 and 120%, respectively. On the basis of the signal to noise ratio value (3:1) obtained in the experiment, the lower limit of detection was five-fold lower and equal to 0.002 mg × mL^{-1}.

High and reproducible recoveries were obtained for all compounds investigated by comparing the peak areas of the extracted samples versus equal amounts of solutions at 0.015, 4 and 80 mg × mL^{-1} concentrations of the analytes. The relative extraction recoveries for PTX and (-)-(R)-M1 from all matrices were from 85.4 ± 4.7% to 112.3 ± 5.6% and from 83.4 ± 3.7% to 114.8 ± 17.8%, respectively. For the IS, at a concentration of 5 mg × mL^{-1}, the mean extraction recovery was found to be 75.1 ± 4.36%. Extraction efficiency was independent of the concentration over the range studied. The liquid-liquid extraction procedure is simpler to develop, requires much less expensive equipment and remains a useful alternative when a large number of samples have to be analyzed.

Over a 6 h period of the short-term stability test, the predicted concentrations for PTX and (-)-(R)-M1 with QC samples deviated within 15% of the nominal concentrations, and no significant degradation could be detected in the samples. The data also reflect the stability of the compounds during the freezing process. PTX and (-)-(R)-M1 were found to be stable in the serum and tissues when stored at – 30 and – 80°C, respectively, for at least four weeks. The results of QC samples following three repeated freeze/thaw cycles have shown that the analytes were stable in the frozen serum at – 30°C and in frozen tissues at – 80°C. The data of stability tests for PTX and (-)-(R)-M1 from serum are presented in Tables 4 and 5, respectively.

For verification of this method for possible application in a pilot pharmacokinetic study of pentoxifylline, PTX was administered intraperitoneally at a dose of 50 mg × kg^{-1} to male Wistar rats. Due to the lack of the reference substance available, the (+)-(S)-M1 enantiomer was quantified in serum and tissues from a calibration curve prepared from the M1 racemic compound.

In the animal's treatment group, PTX, (-)-(R)-M1 and (+)-(S)-M1 concentrations declined over time in a log-linear fashion (Figure 3).

Tissue distribution of the parent drug and the enantiomers of its active metabolite M1 was investigated in liver, lung, kidney, heart and spleen. Figure 4 illustrates the tissue concentration of PTX, (+)-(S)-M1 and (-)-(R)-M1 after pentoxifylline intraperitoneal administration. Significant ($p < 0.05$) differences between serum and tissue levels of PTX, (-)-(R)-M1 and (+)-(S)-M1 were observed. Interestingly, in all studied matrices, the areas under the concentration – time curve calculated for (+)-(S)-M1 were higher than that observed for (-)-(R)-M1.

Tissue to serum $AUC_{0\rightarrow inf}$ ratios for all studied compounds after PTX intraperitoneal dosing are presented in Figure 5. For PTX, these ratios were very low and ranged from 0.12 for the liver to 0.71 for the heart tissue. On the contrary, for (-)-(R)-M1, the tissue to serum $AUC_{0\rightarrow inf}$ ratios were high, with the highest value (32.5) observed in the spleen. In the case of (+)-(S)-M1, the highest tissue to serum $AUC_{0\rightarrow inf}$ values (4.3) were observed in the kidney. Based on the above results, it can be concluded that the developed and validated analytical method allows for the analysis of all compounds of interest in rat biological matrices and is useful for a routine pharmacokinetic study of PTX and its pharmacologically active metabolite, (-)-(R)-M1, in rat.

CONCLUSIONS

We described a simple, sensitive and selective chiral, normal-phase, high-performance liquid chromatography method with UV detection for the analysis of PTX and its pharmacologically active metabolite, (-)-(R)-M1, in rat biological matrices. This method is accurate and suitable for daily direct enantioselective analysis of (-)-(R)-M1 in the presence of PTX. The good sensitivity, separation efficiency and reproducibility of the method were achieved for all biological matrices and allow one to perform pharmacokinetic studies of pentoxifylline in rat matrices. The lower limit of quantification, QC accuracy and precision were well within the range generally accepted for bioanalytical methods. This method was successfully applied to pilot pharmacokinetic studies of PTX and its active metabolite, (-)-(R)-M1, in rats following PTX intraperitoneal dosing.

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